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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	:	Haberbosch, et al.
Serial No.	:	10/655,225
Filed	:	September 4, 2003
For	:	3-DEAZAADENOSIE PREVENTS ATHEROSCLEROSIS AND GRAFT VASCULOPATHY
Art Unit	:	1615
Examiner	:	Carlos A. Azpuru

June 19, 2006

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Eileen Sheffield 7/7/06
Eileen Sheffield

Commissioner for Patents
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Alexandria, Va. 22313-1450

LETTER

Sir:

Attached is an executed Rule 132 Declaration.

If any fees are, authorization is given to charge deposit account no. 50-0624.

Respectfully submitted,

FULBRIGHT & JAWORSKI L.L.P.

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Eileen Sheffield

Commissioner for Patents
P. O. Box 1450
Alexandria, Va. 22313-1450

RULE 132 DECLARATION

Sir:

1. I am the sole inventor of the presently claimed subject matter for the above-identified patent application.

2. I have read and am thoroughly familiar with the specification and pending claims, and I have reviewed the Office Action of January 24, 2006.

3. It is my understanding that the Examiner has made enablement rejections of claims, in particular with respect to the term "prevention" in claims 21, 22, 32, 34, 38 and 40.

4. I have conducted a study, or have had a study conducted at my direction, that provides data showing that methods of prevention of diseases can be achieved according to the claims invention.

5. Attached is an unpublished article that was submitted to a journal for publication entitled 3-Deazaadenosine Prevents Proliferation of Vascular Smooth Muscle Cells and Neointima Formation. The article also names, as my co-authors, Daniel G. Sedding, Monique Tröbs, Fabian Reich, Gerhard Walker, Ludger Fink, Klaus T. Preissner, Rainer Maria M. Bohle, and Alexander C. Langheinrich, each of whom contributed to preparation of the article.

6. The study set forth in the article therein was conducted by me and/or at my direction to provide additional evidence supporting my invention. Certain co-authors named in the article, and others, assisted in the study under my direction and guidance.

7. The study and data derived therefrom, as presented in the article, clearly show that 3-deazaadenosine (c3Ado) is a potent inhibitor of S-adenosylhomocysteine (SAH)-hydrolase and that such inhibition prevents vascular smooth muscle cell proliferation and the development of post-angioplasty restenosis.

8. In the study, it was shown that c3Ado prevents vascular smooth muscle cell proliferation and migration. The results of this experiment are shown, e.g., in Figure 1A which demonstrates that c3Ado prevents an increase in vasular smooth muscle cell (VSMC) numbers in a dose-dependent matter.

9. This study also demonstrates that c3Ado prevents vascular smooth muscle cell proliferation by interfering with Ras signaling. The data indicates that c3Ado prevents Ras translocation from the cytosol to the cell membrane which is the prerequisite for Ras signaling, as determined by immunohistochemistry as shown in Figure 5A. Membrane fractionation from whole cell lysates and immunoblotting shows that the growth-factor induced localization of Ras in the membrane fraction is prevented by c3Ado in a dose-dependent manner (see Figure 5B). From these data, it is clear that c3Ado dose-dependently inhibits vascular smooth muscle cell proliferation and that this was not due to an apoptotic or toxic effect of c3Ado, it is due to the interference of c3Ado with Ras methylation and thus with the Ras downstream signaling in response to mytogenic stimuli.

10. In conclusion, the data presented in this article provide further evidence supporting my claimed invention that c3Ado can prevent, as well as treat, vascular disease.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

By---
Name Werner Haberbosch

21.05.06
Date

3-Deazaadenosine Prevents Proliferation of Vascular Smooth Muscle Cells and Neointima Formation

Short title: C3Ado prevents Neointima Formation

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ABSTRACT

Background: 3-Deazaadenosine (c3Ado) is a potent inhibitor of S-adenosylhomocysteine (SAH)-hydrolase which regulates cellular methyltransferase activity. In the present study we sought to determine c3Ado's effect on vascular smooth muscle cell (VSMC) function and neointima formation in vivo.

Material and Methods: C3Ado dose-dependently prevented the proliferation and migration of human coronary VSMC in vitro. This was accompanied by an increased expression of the cyclin-dependend kinase inhibitors $p21^{WAF1/Cip1}$, $p27^{Kip1}$, a decreased expression of G1/S-phase cyclins and a lacking retinoblastoma protein hyperphosphorylation. FACS analysis of propidium iodide stained cells indicated a cell cycle arrest in the G0/G1 phase. Rescuing signal transduction by overexpression of a constitutive active Ras mutant abrogated c3Ado's effect on proliferation. For in vivo studies, the femoral artery of C57BL/6 mice was dilated and mice received a diet containing 150 µg c3Ado/day. c3Ado prevented dilation-induced Ras-activation as well as ERK 1/2 and Akt phosphorylation in vivo. At day 21, VSMC proliferation (7.9 ± 0.7 % vs. 10.8 ± 0.8 % PCNA pos. cells $P<0.05$) as well as the neointima/media ratio (0.7 ± 0.2 vs. 1.6 ± 0.4; $P<0.05$) were significantly reduced.

Conclusion: Our data indicate that c3Ado interferes with Ras methylation and function and thereby with mitogenic activation of ERK 1/2 and Akt, preventing VSMC cell cycle entry and proliferation and neointima formation in vivo. Thus, therapeutic inhibition of SAH-hydrolase by c3Ado may represent a novel approach to prevent vascular proliferative disease.

Condensed abstract

3-Deazaadenosine (c3Ado), a potent inhibitor of S-adenosylhomocysteine hydrolase inhibits Ras signaling by interfering with its post translational methylation. C3Ado thereby inhibits cell cycle entry, proliferation and migration of vascular smooth muscle cells without exerting cytopathic effects. Moreover, c3Ado effectively reduces neointima formation in a mouse model of vascular injury. Therefore, prevention of Ras methylation by c3Ado may represent a novel therapeutic strategy for the treatment of vascular proliferative diseases.

INTRODUCTION

Vascular smooth muscle cell (VSMC) migration, proliferation, and hypertrophy triggered by inflammatory responses of the vessel wall are considered to be key events in the development of atherosclerosis, postangioplasty restenosis, and venous bypass graft failure.[1, 2] Consequently, anti-inflammatory and anti-proliferative[3] strategies have been demonstrated to successfully prevent the development of vascular proliferative disease. Therefore, the identification of novel compounds with combined anti-inflammatory/antiproliferative properties holds promise to improve current therapeutic strategies by limiting late complications like in-stent restenosis or bypass graft failure.

3-Deazaadenosine (c3Ado), a structural analogue of adenosine with no adenosine-receptor interaction is a potent inhibitor of S-adenosylhomocysteine hydrolase which regulates cellular methyltransferase activity. c3Ado has previously been shown to inhibit a variety of cellular functions, which might be critical for the development of atherosclerosis and restenosis, i.e. thrombin-stimulated production of platelet-derived growth factor and the expression of endothelial leukocyte adhesion molecule-1[4] as well as cellular arachidonic acid and ROS production.[5, 6] Moreover, c3Ado prevents TNF-α production, reduces TNF-α-induced macrophage adhesion to endothelial cells in vitro via the inhibition of ICAM-1 synthesis, and promotes monocyte apoptosis.[7] We recently demonstrated that c3Ado inhibits endothelial expression of ICAM-1 and VCAM-1 in vivo and prevents diet-induced plaque formation in apo$E^{-/-}$ mice.[8] However, despite its potent anti-inflammatory properties, the molecular mechanism of c3Ado's action is not well understood.

Besides inflammatory responses, the proliferation of VSMC comprises a major determinant in the development of atherosclerosis and restenosis.[2] It is, however, unclear if c3Ado, in addition to its anti-inflammatory properties, has a direct effect on cell cycle progression and

proliferation of VSMC and thus might be a suitable compound to even prevent highly proliferative vascular responses like post-angioplasty restenosis.

Thus, we evaluated the molecular mechanisms of c3Ado's action and aimed to analyze the effect of c3Ado on VSMC proliferation and migration and its impact on neointima formation. By inhibiting S-adenosylhomocysteine hydrolase, c3Ado regulates cellular S-adenosylhomocysteine (SAH) levels. Because elevated intracellular concentration of SAH results in product inhibition of S-adenosylmethionine (SAM)-dependent methyltransferases,[9] we hypothesized that inhibition of methyltransferase activity mediates the c3Ado-dependent modulation of VSMC function. Since post-translational methylation is an important step in the activation of CAAX sequence containing signaling proteins such as the Ras superfamily of GTPases, we hypothesized that c3Ado may interfere with Ras function and subsequent downstream signaling regulating VSMC cell cycle progression and proliferation.

Our data demonstrate that by exerting potent anti-proliferative and anti-inflammatory properties, therapeutic inhibition of increased signaling protein methylation in activated VSMC by c3Ado may represent a novel approach to prevent vascular proliferative disease.

METHODS

Quantification of VSMC Proliferation

VSMC were detached using 1 mmol/L Trypsin (Gibco), harvested by centrifugation, resuspended in 500 µL of supplemented growth medium containing 5% FCS (SmGM-2; Clonetics, Verviers, Belgium), counted, and reseeded in 24 well culture dishes. Different concentrations of c3Ado were added to the growth medium. After 48 hours of incubation at 37°C, 5% CO_2, cells were trypsinized and counted using a hemocytometer.

Alternatively, BrdU incorporation was determinded according to the supplier's instructions (In situ cell death detection kit, Roche, Mannheim, Germany).

Quantification of VSMC Migration

VSMC were detached using 1 mmol/L Trypsin (Gibco), harvested by centrifugation, resuspended in 500 µL of SmGM-2, counted, and placed in the upper chamber of a modified Boyden chamber. The chamber was placed in a 24-well culture dish containing SmGM-2 and human recombinant PDGF (20 ng/mL). After 6 hours of incubation at 37°C, 5% CO_2, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cell nuclei were stained with trypan blue. Migrating cells into the lower chamber were counted in three high power fields.

Quantification of VSMC Apoptosis

VSMC were cultured in the absence or presence of different concentrations of c3Ado for 24 h. VSMC were fixed using 2% paraformaldehyde, and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) was performed according to the supplier's instructions (In situ cell death detection kit, Roche, Mannheim, Germany). After staining all nuclei with DAPI, samples were viewed with an inverted fluorescence microscope (Leica) and two independent investigators, blinded to the treatment, evaluated the relative number of apoptotic cells per well by counting 4 randomly selected high-power fields.

Trypan blue staining

Trypan blue exclusion was used to determine the viability of VSMC. VSMC were trypsinized after addition of c3Ado for 24h and incubated with 0.4% trypan blue dye for 2 min. VSMC were observed with the use of a hemocytometer under a light microscope. Cells that were able to exclude the stain were considered viable and the percentage of nonblue cells over total cell number was used as an index of viability.

Flow Cytometry (Cell Cycle Distribution)

Cells were harvested by trypsinization, fixed overnight with 75 % methanol, washed, and incubated with 100 μg/ml RNase (Oncogene Research Products, Cambridge, Massachusetts) and 10 μg/ml propidium iodide in PBS for 1 h at 37 °C. Samples were analyzed for DNA content using a high speed cell sorter (EPICs Altra, Beckman Coulter, Miami, Florida). Data were computer-analyzed with a commercially available software (Multicycle, Phoenix Flow Systems, San Diego, California).

Transfection Procedures

For transfection and magnetic activated cell sorting (MACS) to enrich for positively transfected cells, cells were co-transfected with equimolar amounts of pMACS.K^k-II and the expression plasmid in a 1:3 ratio using FuGENE 6 Transfection reagent (Roche, Mannheim, Germany) as described previously.[10]

Preparation of Cellular Lysates and Immunoblot Analysis

Semiquantitative analysis of proteins in cell lysates was performed by western blotting and antibody detection as previously described.[11] Briefly, the cleared supernatant from lysates was run on polyacrylamide gel, and blotted onto nitrocellulose (Hybond-ECL, Amersham, Freiburg, Germany) by wet electroblotting. After blocking, blots were incubated with primary antibody (for detailed description of the antibodies used please refer to the online materials and methods section) for 1 h at room temperature. Proteins were then detected by enhanced

chemiluminescence (ECL+, Amersham) after labelling with horseradish peroxidase-labelled secondary antibody (1:2000 for 1 h) according to the manufacturer's instructions.

Ras Activity Assay

VSMC were grown to near confluence, serum-starved and incubated with c3Ado at the indicated concentrations for 24 h, and stimulated with VSMC growth medium for 10 min, all at 37 °C, 5% CO_2. Cells were lysed and centrifuged, and equivalent amounts (0.5 mg/experiment) of supernatant protein from each condition were assayed for active Ras (Ras-GTP) according to the supplier's instructions (Upstate, Lake Placid, NY). Alternatively, and for in vivo experiments, a more sensitive ELISA-based Ras activity assay, requiring less amounts of protein, was performed according to the supplier's instructions (Ras GTPase Chemi ELISA kit, Active Motif, Rixensart, Belgium).

Mouse Femoral Artery Angioplasty

Male C57/BL6 mice (Charles River, Quebec, Canada) were anesthetized with 150 mg/kg body weight (bw) ketaminehydrochloride (Ketanest, Pharmacia/Pfizer, Mannheim, Germany) and 0.1 mg/kg bw xylazinehydrochloride (Rompun 2%, Bayer) underwent transluminal mechanical injury of the left femoral artery by insertion of a straight spring wire (0.38 mm in diameter; Cook, Bloomington, IN) for >5 mm towards the iliac artery, as previously described.[12] All procedures involving experimental animals were approved by the institutional committee for animal research of the Giessen University and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985).

Morphometric Analysis

Samples were sectioned on a Leica cryostat (6 µm) and placed on poly-L-lysine (Sigma)–coated slides for immunohistochemical analysis. For morphometric analyses, hematoxylin and eosin staining was performed according to standard protocols. All sections were

examined under a Leica DMRB microscope. For morphometric analyses, KS300 software (Carl Zeiss, Hallbergmoos, Germany) was used to measure external elastic lamina, internal elastic lamina, and lumen circumference, as well as medial and neointimal area of 6 sections per animal.

Immunohistochemistry

Cross sections of mouse femoral arteries were fixed for 10 min in freshly prepared, buffered 4 % paraformaldehyde. Sections were covered for 20 min with 10% normal goat serum, followed by incubation with rabbit polyclonal anti-vWF (1:500, Sigma, Taufkirchen, Germany) or mouse monoclonal anti-Ras (1:200, Upstate, Lake Placid, NY) and PE conjugated mouse monoclonal anti-smooth muscle actin (sma, 1:500, Sigma) for 1 h in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. After two washing steps (10 min in PBS), cross sections were incubated with secondary antibodies for 40 min: donkey anti-rabbit IgG or donkey anti-mouse conjugated to Alexa Fluor® 488 (1:200, Molecular Probes, Leyden, The Netherlands). After washing, slides were mounted in Vectashield® mounting medium H-1000 containing 4',6-Diamidino-2-phenylindole, 2HCl (DAPI, 5 µg/ml, Linaris, Wertheim, Germany) and evaluated using an epifluorescence microscope (DMRB, Leica, Wetzlar, Germany). Negative controls were performed using an unspecific primary antibody or the secondary antibody only. For the detection of VSMC proliferation in vivo, PCNA staining was performed according to the supplier's instructions (PCNA staining kit, Zymed - Invitrogen, Karlsruhe, Germany).

Statistical Analyses

Data were stored and analyzed on personal computers using Excel 2000 (Microsoft) and Sigma Stat 2.03 (Systat, Erkrath, Germany). Data between the study groups were analyzed by 1-way ANOVA followed by pair wise multi comparison using the Holm-Sidak method. All

data are represented as mean ± standard error of the mean (SEM). A probability value <0.05 was considered statistically significant for all comparisons.

For further descriptions of Materials and Methods please refer to the supplemental methods section.

RESULTS

C3Ado prevents VSMC Proliferation and Migration

To evaluate the effect of c3Ado on VSMC function, VSMC were grown in growth medium containing 5% FCS in the absence or presence of different concentrations of c3Ado. C3Ado prevented the increase in VSMC cell numbers in a dose dependent manner (51.6 ± 40.8 % vs 198.6 ± 35.0 % increase in cell number after 48 h, n = 4, $p < 0.05$ at 50 μM c3Ado; **Figure 1A**). The reduced increase in cell numbers was due to an inhibition of VSMC proliferation. **Figure 2B** illustrates results from a BrdU incorporation assay and shows that DNA replication is inhibited by 60% in VSMC treated with 50 μM c3Ado compared to controls (n = 4, $p < 0.05$). Furthermore, c3Ado dose-dependently prevented VSMC migration (28.8 ± 2.3 vs. 73.8 ± 9.6 cells/ high power field, n = 4, $p < 0.05$ at 50 μM c3Ado; **Figure 1C**).

Effect of c3Ado on VSMC Apoptosis and Necrosis

To exclude that the lack of increase in cell numbers is also due to an apoptotic effect of c3Ado, we determined the number of apoptotic cells in the absence or presence of c3Ado. As shown in **Figure 2A**, c3Ado did not induce apoptosis (TUNEL positive cells), even at concentrations of 100 μM (1.6 ± 0.6 % vs 2.2 ± 0.8 % of total cells, n = 4, p = n.s. at 100 μM c3Ado). There was also no effect of c3Ado on cell necrosis as determined by Trypan blue exclusion (80.9 ± 8.8 % vs 81.4 ± 6.2 % viable cells, n = 4, p = n.s. at 100 μM c3Ado; **Figure 2B**). Furthermore, the anti-proliferative effect of c3Ado was reversible: Cells were serum starved and synchronized in the presence or absence of c3Ado for 24 h. After three washing steps, cells were incubated in basal medium for 12 h, then in growth medium containing BrdU for 24 h and BrdU-incorporation was quantified. Cells pretreated with c3Ado showed an almost similar proliferation rate as cells pretreated with a control buffer only. In contrast, cells pretreated with actinomycin D did not proliferate in the presence of growth medium (n = 4;

Figure 2C). These data indicate that there is no toxic or apoptotic effect of c3Ado in the concentrations tested, which could account for c3Ado's effect on proliferation and migration.

C3Ado Prevents Cell Cyle Entry / Progression in the G0/G1 phase

To elucidate the mechanisms responsible for the anti-proliferative effect of c3Ado, we determined the cell cycle progression and expression of cell cycle regulating proteins in the absence or presence of c3Ado. As determined by FACS analysis, c3Ado dose-dependently prevented the cell cycle entry/-progression in the G0/G1 phase, indicating that c3Ado interferes with very early processes of cell cycle progression (**Figure 3A+B**). c3Ado dose-dependently prevented the downregulation of the cyclin-dependent kinase inhibitors (CDKI) $p21^{cip1}$ and $p27^{Kip1}$ as well as the upregulation of cyclin A, cyclin B, cyclin D and cyclin E, which are essential for the progression through the G0/G1 phase. A block in G0/G1 phase was also confirmed by the lacking hyper-phosphorylation of the retinoblastoma gene product (RB) (**Figure 3C**). However, there was no specific regulation of one of these molecules, suggesting that c3Ado may not affect the expression or function of a single cell cycle regulatory protein but rather may interfere with upstream signaling mechanisms that regulate early cell cycle entry.

Effect of c3Ado on Akt and Erk Activation

Since the MAPK-pathway as well as the PI3-K/Akt signal transduction pathway are known to trigger early cell cycle events like the expression of the CDKIs $p21^{cip/waf}$ and $p27^{Kip1}$or the expression of cyclins, we evaluated the effect of c3Ado on the serum-induced activation of these pathways. Surprisingly, c3Ado did interfere with the serum-induced activation of both pathways. C3Ado dose dependently prevented the phosphorylation of Raf and ERK (**Figure 4A**). c3Ado also dose-dependently prevented the phosphorylation of the protein-dependent kinase 1 (PDK1), of the protein kinase B (Akt) and of the forkhead transcription factor

FoxO1a, known to regulate the expression of $p27^{kip1}$ and D-type cyclins (**Figure 4B**). These data indicate, that c3Ado may interfere with processes upstream of the serum-induced activation of these signal transduction pathways.

C3Ado Prevents VSMC Proliferation by Interfering with Ras signaling

The GTPase Ras has been described to transduce growth factor-induced tyrosine kinase signaling towards ERK and Akt activation. Furthermore, Ras translocation to the membrane and activation is dependent on its carboxyl methylation by methyltransferases which were shown to be inhibited by c3Ado. Therefore, we sought to determine whether c3Ado may prevent growth factor-induced ERK and Akt activation by interfering with Ras signaling. Our data indicate that c3Ado prevents Ras translocation from the cytosol to the cell membrane, a prerequisite for Ras signaling, as determined by immunohistochemistry (**Figure 5A**). As shown by membrane fractionation from whole cell lysates and immunoblotting using specific antibodies, the growth factor-induced localization of Ras in the membrane fraction is prevented by c3Ado in a dose-dependent manner (**Figure 5B**). In accordance with these data, the growth factor-induced Ras activation (Ras-GTP) was also reduced in a dose-dependent manner (**Figure 5C**). To test whether the inhibitory effect on Ras activity is responsible for the anti-proliferative effect of c3Ado, a constitutively active mutant of Ras [Ras(+)] was overexpressed in VSMC. (Note that only positively transfected cells were sorted and evaluated as described in the methods section). Cells overexpressing the constitutive active form of Ras were resistant to the c3Ado-induced antiproliferative effect, indicating that c3Ado interferes with VSMC proliferation mainly by preventing Ras activation and Ras-dependent signal transduction (**Figure 5D**).

C3Ado-dependent inhibition of Ras carboxyl methylation, activation and signaling is dependent on ICMT

We hypothesized that inhibition of isoprenylcysteine carboxyl methyltransferase (ICMT) by c3Ado would inhibit Ras activation by preventing the methylation of its carboxy terminal CAAX motive. We therefore assessed Ras activity in cells treated with c3Ado, the ICMT inhibitor AGGC (20 µM), or the inactive analogue AGC (20 µM). Ras activity was significantly decreased after incubation of VSMC with AGGC as it was after incubation with c3Ado (**Figure 6A**). Consistently, treatment with the ICMT inhibitor AGGC significantly prevented VSMC proliferation to the same extent as c3Ado compared with the inactive analogue AGC (**Figure 6C**). Overexpression of ICMT prevented the c3Ado-dependent inhibition of Ras activity (**Figure 6B**), indicating that c3Ado interferes with Ras activity by inhibiting ICMT methyltransferase activity. Furthermore, the AGGC-mediated inhibition of proliferation was abrogated when Ras activity was reconstituted by overexpression of a constitutively active Ras mutant (**Figure 6D**). Taken together, these data demonstrate that c3Ado inhibits Ras methylation and signaling by interfering with ICMT methyl transferase activity and that inhibition of Ras represents a key mechanism of c3Ado's anti-proliferative effect.

c3Ado Prevents Neointima Formation in Vivo

To test the effect of c3Ado in vivo, mice were fed with the indicated concentrations of c3Ado for two days. Ras activity and Akt / ERK phosphorylation were determined in arteries 20 min after dilation. As indicated in **Figure 7A+B**, pretreatment of mice with c3Ado reduced dilation-induced Ras activation and Akt and ERK phosphorylation. To evaluate the effect of c3Ado on neointima formation, mouse femoral arteries were excised 21 days after dilation and morphometric analysis was performed. A significant concentric neointima is evident, with the media clearly defined by the internal and external elastic laminae. The middle and right pictures show injured femoral arteries from mice treated with 75 µg c3Ado/day or 150 µg c3Ado/day respectively (**Figure 7C**). Intima/media ratio (I/M) and neointimal area were

significantly reduced in c3Ado treated femoral arteries compared to control vessels (Neointima/media ratio: 0.7 ± 0.2 [150 μg c3Ado/day] vs. 1.6 ± 0.4 [control diet]; n=6; $P<0.05$; **Figure 7D**). Medial wall area was not significantly reduced in the c3Ado treated groups (**Figure 7D**), which is in accordance to the non-apoptotic/non-toxic effect of c3Ado seen in vitro.

Effect of c3Ado on VSMC Proliferation, Apoptosis, and Re-endothelialization in vivo

Further immunohistochemical evaluation of neointimal tissues revealed that c3Ado's potency to prevent neointima formation results from an anti-proliferative effect as determined by quantification of PCNA-positive cells 21 days following injury (6.7 ± 2 % vs. 10.8 ± 0.8 % PCNA positive cells in arteries from mice treated with 150 μg c3Ado/day, $p < 0.05$; **Fig. 8A**). Complementing the in vitro data, c3Ado did not augment apoptosis of VSMC as determined by quantification of TUNEL-positive cells (1.8 ± 0.6 % vs. 1.6 ± 0.4 % TUNEL positive cells in arteries from mice treated with 150 μg c3Ado/day, $p < 0.05$; **Fig. 8B**). Furthermore, treatment of mice with c3Ado at different concentrations had no effect on re-endothelialization of vessels 21 days after dilation as determined by immunohistochemical detection of CD31+ cells lining the luminal side of the neointima (**Fig. 8C**).

DISCUSSION

Vascular smooth muscle cells play a prominent role in the pathogenesis of vascular proliferative disorders such as atherosclerosis, postangioplasty restenosis, bypass vein graft failure, and cardiac allograft vasculopathy.[2] Identification of the key mechanisms involved in VSMC function will help to understand cellular responses to vascular injury. Moreover, the identification and characterization of small molecules interfering with these pathways will help to develop safe and efficient therapeutic strategies for the prevention of vascular proliferative disease.

In the present study we demonstrate that inhibition of SAH-hydrolase by c3Ado, a structural analogue of adenosine with no adenosine-receptor interaction, prevents VSMC proliferation and the development of postangioplasty restenosis. We provide evidence that c3Ado inhibits Ras methylation and thereby interferes with Ras downstream signaling in response to mitogenic stimuli. c3Ado thereby prevents the activation of the Akt as well as the ERK pathway, both known to be essential for the induction of cell cycle entry. Indeed, treatment with c3Ado resulted in a stabilization of the cyclin-dependent kinase inhibitors (CDKI) $p21^{cip/waf1}$ and $p27^{Kip1}$ while preventing the transcriptional activation of G1-phase cyclins. Subsequently, c3Ado prevented G1/S phase transition and proliferation of VSMC. Furthermore, c3Ado inhibited migration without promoting apoptosis of VSMC. The efficiency of c3Ado was also observed in vivo, since oral administration of c3Ado effectively prevented neointima formation in a murine model of wire-induced vascular injury.

In initial experiments, we evaluated the role of c3Ado on VSMC proliferation. Our data demonstrate, that c3Ado dose-dependently inhibited VSMC proliferation. Importantly, this was not due to an apoptotic or toxic effect of c3Ado. These findings are in accordance with previous reports which described a potent anti-proliferative effect of SAH-hydrolase inhibition on vascular cells[13], indicating that inhibition of methyltransferase activity represents a potentially interesting target for anti-proliferative strategies.

However, the molecular mechanism of c3Ado-dependent regulation of VSMC proliferation still remained elusive. We therefore used FACS analysis to determine the cell cycle distribution of c3Ado treated cells. Our results indicated that c3Ado inhibits cell cycle progression very early in G0/G1 phase, however, c3Ado did not seem to specifically regulate the expression of the tested G0/G1 phase proteins. We therefore hypothesized that c3Ado interferes with upstream signaling regulating G0/G1 phase progression.

We and others previously demonstrated that the mitogen-activated protein kinase (MAPK;ERK) and PI3-K/Akt pathway are essential for proper cell cycle entry and progression through G0/G1 phase and proliferation of VSMC.[14] We therefore tested whether c3Ado might interfere with Akt or ERK signaling or other signaling pathways (data not shown) regulating G0/G1 progression. Our experiments indicate that c3Ado dose-dependently inhibited the phosphorylation and activation of both, the PI3-K/Akt and ERK pathway, suggesting that c3Ado might interfere with methylation-dependent signaling processes upstream of Akt and ERK.

The activation of ERK and Akt is strongly dependent on the GTPase Ras which regulates a wide variety of cellular functions, including growth, differentiation, and apoptosis. To become active, Ras GTPase proteins must associate with cellular membranes.[15-17] However, Ras plasma membrane association requires a series of post-translational modifications of its carboxyl terminus including farnesylation, proteolysis, and methylation of its CAAX sequence by an isoprenylcysteine carboxyl methyltransferase (ICMT).[18-20] Methylation of CAAX proteins has been shown to be stimulated by various inflammatory and mitogenic stimuli such as TNFa,[21] high glucose,[22] and *N*-formyl-Met-Leu-Phe.[23] Thus, methylation appears to be a common mechanism by which various activators stimulate the activity of signaling proteins. Moreover, the importance of ICMT-dependent methylation was recently

demonstrated by the finding that ICMT-deficient mice did not survive beyond mid-gestation.[24]

However, ICMT requires S-adenosyl methionine (SAM) as a methyl group donor to produce methylated Ras, resulting in the accumulation of S-adenosyl homocysteine (SAH). SAH is broken down by SAH-hydrolase into adenosine and homocysteine. However, inhibition of the SAH-hydrolase results in the accumulation of SAH, which then, by product inhibition, acts as a potent inhibitor of SAM-dependent ICMT activation and subsequently of Ras function.[25] Therefore, by inhibiting SAH-hydrolase, c3Ado may prevent the increased methylation and activation of signaling molecules in activated VSMC.

Our data demonstrate that c3Ado dose-dependently inhibited growth factor-induced Ras membrane association and activity. Furthermore, we provide evidence that inhibition of Ras signaling is one of the key mechanisms of c3Ado's anti-proliferative effect in VSMC, since overexpression of a constitutive active Ras mutant almost completely rescued VSMC from c3Ado's antiproliferative effect. Furthermore, our data indicate that c3Ado regulates Ras methylation via the inhibition of ICMT: Since ICMT inhibitors prevented Ras activation and proliferation to the same extent as c3Ado, the overexpression of ICMT restored serum-induced Ras activation in the presence of c3Ado. These data are in accordance with results previously described by Wang *et al.*[13] who demonstrated that co-incubation of endothelial cells with homocysteine and an adenosine deaminase inhibitor, a strategy that inhibits SAH-hydrolase and thereby ICMT activity, decreased the level of carboxyl methylation and plasma membrane localization of v-H-Ras as well as endothelial cell proliferation.

Recently, a role for Ras-GTPase in vascular homeostasis and disease development has been suggested: Dependency of p42/p44 MAPK activation on Ras-GTPase has been demonstrated in vivo in a porcine model of balloon injury early after angioplasty.[26] Moreover, the local delivery of H-*ras* dominant negative mutant (N17 and L61, S186) plasmid constructs,[27]

adenovirus-mediated transfer of dominant negative H-*ras*[28] or prevention of posttranslational modification by local delivery of a Ras farnesyl transferase inhibitor[29] have been shown to prevent ERK activation and to significantly reduce neointima formation. Recent findings, however, indicate that the inhibition of the post-prenylation-processing steps, particularly that of ICMT-catalyzed methylation of Ras might provide the most effective approach to control cell proliferation.[30] Accordingly, our data indicate that c3Ado effectively inhibited Ras activation in vivo following wire-induced injury of the mouse femoral artery. Moreover, c3Ado effectively reduced neointima formation in injured arteries, which was due to an inhibition of VSMC proliferation in vivo.

Interestingly, a recent study demonstrated that SAH-hydrolase- inhibition resulting in impaired Ras signaling is also a critical component of the anti-proliferative effect of methotrexate, one of the most successful drugs in cancer therapy,[31] which was also successfully used to prevent in-stent restenosis in a porcine coronary artery model.[32] However, beside c3Ado's anti-proliferative effect on VSMC, c3Ado's previously described inhibitory effects on ROS production,[6] adhesion molecule expression,[4, 8] leukocyte recruitment and monocyte- and T cell activation[33] may further contribute to the effective reduction of vascular lesion formation.

Our experiments indicate that c3Ado attenuates VSMC migration as well. Since VSMC migration is a key event in neointima development,[1] prevention of cell migration by c3Ado may additionally contribute to reduced lesion formation in vivo. Previously, Harrington *et al.* reported, that SAH hydrolase-inhibition by adenosine and homocysteine interferes with the assembly of focal adhesion complexes[34] which are essential for VSMC proliferation. Furthermore, a direct requirement of Ras signaling for VSMC migration was described.[35] However, the anti-migratory effect might also be a general response to cell cycle arrest rather than a c3Ado specific effect, since cell cycle inhibition prevents not only cellular proliferation

but can modulate numerous cellular functions like differentiation, inflammation and migration.[14] Yet, the specific role of c3Ado in the regulation of cell migration needs further investigation.

Previous studies reported that inhibition of ICMT and subsequent Ras signaling may cause apoptosis of pulmonary artery endothelial cells.[36] Furthermore, the anti-apoptotic effect of Ras-dependent Akt activation was described in a wide variety of cell types. Given the concerns that c3Ado might also modulate endothelial cell apoptosis and proliferation, we assessed the re-endothelialization of denuded vessels. However, we did not observe any differences in re-endothelialization 4 weeks after injury, suggesting that therapeutic application of c3Ado will not be accompanied by an increased risk of target vessel thrombosis. Moreover, our data indicate that c3Ado does not induce apoptosis of VSMC in vitro or in vivo even at the highest concentrations tested. Furthermore, there was no toxic effect of c3Ado as demonstrated by trypan blue exclusion and the reversibility of c3Ado's anti-proliferative action in vitro and as supported by the in vivo findings showing no thinning of the medial wall. These findings are in accordance with previous reports, showing that a specific, reversible type III SAH-hydrolase inhibitor (DZ2002) did not exert any toxic effects despite it's potent inhibitory potential,[33] and that c3Ado prevented thrombin-dependent endothelial cell activation without inducing cytopathic effects.[4] Therefore, in regard to toxicity and safety of a potential therapeutic application, SAH-hydrolase inhibition by c3Ado might be superior to directly targeting Ras signaling. Importantly, our data suggest that by interfering with VSMC proliferation in acute or early lesions and by interfering with inflammatory responses without inducing apoptosis of vascular cells in stable lesions, c3Ado may represent an attractive molecule for the prevention of acute as well as chronic vascular disease.

In conclusion, our data indicate that the prevention of (increased) methylation of signaling proteins by c3Ado, which inhibits SAH-hydrolase, ICMT and subsequent Ras signaling, may represent an attractive therapeutic strategy for the prevention of vascular proliferative diseases.

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Figure 1. Effect of c3Ado on VSMC proliferation and migration. **A**, VSMC were incubated in growth medium containing 5% FCS in the absence or presence of different concentrations of c3Ado and total cell number was evaluated after 48 h (*$P< 0.05$, n= 4). **B**, VSMC were incubated in growth medium containing 5% FCS in the absence or presence of different concentrations of c3Ado for 24 h in the presence of BrdU. VSMC proliferation is expressed as mean OD450 ± SEM as determined by anti-BrdU ELISA (*$P< 0.05$, n= 4). **C**, VSMC were added to the upper side of gelatin-coated tissue culture inserts and allowed to migrate for 6 hours in the presence or absence of PDGF (20 ng/ml) or different concentrations of c3Ado to the lower chambers. After microscopic evaluation of inserts, the number of migrated cells was expressed as cells per high power field (cells/HPF).(*$P< 0.05$, n= 4).

Figure 2. **A**, VSMC were incubated in growth medium containing 5% FCS in the absence or presence of different concentrations of c3Ado for 24 h and the relative number of apoptotic cells was evaluated at the indicated time points by TUNEL staining (P=n.s., n= 4). **B**, VSMC were incubated in growth medium containing 5% FCS in the absence or presence of different concentrations of c3Ado for 24 h and cell viability was evaluated after trypsinisation by counting the relative number of cells that excluded the trypan blue dye (P=n.s., n=4). **C**, VSMC were incubated in growth medium containing 5% FCS in the absence or presence of c3Ado for 24 h. Following three washes, cells were incubated in growth medium for 24 h and VSMC proliferation was quantified by BrdU incorporation (P=n.s., n=4).

Figure 3. c3Ado modulates cell cycle progression in VSMC. **A**, representative graphs of cell cycle distribution determined by flow-cytometric evaluation are shown. **B**, Quantification of VSMC in G0/G1- S- or G2/M-phase determined by flow-cytometric evaluation is demonstrated (black bars = serum-withdrawn, synchronized cells; white bars = proliferating cells after exposure to growth medium for 24 h ; grey bars = proliferating cells in the presence of growth medium and different concentrations of c3Ado; 10 μM, 50 μM and 100 μM; n= 4;

*$P < 0.05$). **C**, VSMC in the absence or presence of different concentrations of c3Ado were incubated in growth medium or basal medium for 24 hours and expression of cell cycle proteins was determined by western blotting using specific antibodies. Detection of Cdk4 served as a loading control.

Figure 4. c3Ado prevents the growth factor-induced activation of Erk and Akt. **A**, VSMC in the absence or presence of different concentrations of c3Ado were incubated in growth medium or basal medium for 24 hours and phosphorylation of different molecules in the Erk and PI3-K/Akt signal transduction cascade was determined by immunoblotting using specific antibodies. CDK4 served as control for equal protein loading.

Figure 5. Effect of c3Ado on Ras localization, translocation and activation. **A**, Representative high-magnification pictures of VSMC that were treated as indicated and stained for Ras (green) and nuclei (DAPI, blue) are shown. **B**, VSMC were treated as indicated and membrane translocation of Ras was determined after subcellular fragmentation by immunoblotting using specific antibodies. Adox, another SAH-hydrolase-inhibitor was added as assay control. **C**, Ras-GTP (active Ras) was immunoprecipitated using agarose-bound Raf-1 RBD and the amount of activated Ras was determined by immunoblotting using specific antibodies. Adox, another SAH-hydrolase-inhibitor was added as assay control. Detection of total Ras in cell lysates using specific antibodies served as control for equal protein loading. **D**, VSMC were transfected with a constitutive active Ras (Ras+) or a control plasmid and proliferation of VSMC in the presence or absence of c3Ado (50 µM) was determined by BrdU-incorporation.

Figure 6. c3Ado interferes with Ras signaling by inhibiting ICMT methyl transferase activity. **A**, Ras activity was determined by an ELISA-based activity assay and results are expressed as relative light units (RLU;*$P < 0.05$, n= 4). **B**, VSMC were transfected with ICMT (white bars) or a control plasmid expressing GFP (black bars) and Ras activity in the presence or absence

of c3Ado (50 μM) was determined by Ras activity assay (*P< 0.05, n= 4). C, VSMC were incubated in growth medium containing 5% FCS in the absence or presence of different concentrations of c3Ado or the ICMT inhibitor AGGC or its inactive analogue AGC for 24 h and total cell number was evaluated after 48 h (*P< 0.05, n= 4). D, VSMC were transfected with a constitutive active Ras (Ras+) or a control plasmid (GFP) and proliferation of VSMC in the presence or absence of the ICMT inhibitor AGGC or its inactive analogue AGC was determined by BrdU-incorporation (*P< 0.05, n= 4).

Figure 7. C3Ado prevents neointima formation in vivo. **A**, quantification of Ras activity following dilatation of the mouse femoral artery in the presence or absence of c3Ado. **B**, Phosphorylation of Akt and ERK was determined by immunoblotting of lysates from mouse femoral arteries following dilation in the presence or absence of c3Ado. Detection of actin served as a loading control. **C**, Representative cross sections of femoral arteries from mice fed with a control diet only (left), a diet containing 75 μg c3Ado/d (middle) or 150 μg c3Ado/d (right) 4 weeks after dilatation are shown. **D**, Quantification of neointima/media (I/M) ratio, intimal area, medial area, and luminal area of femoral arteries from mice fed with a control diet only (left), a diet containing 75 μg c3Ado/d (middle) or 150 μg c3Ado/d (right) 4 weeks after dilatation are shown (n= 6, *P< 0.05).

Figure 8. Effect of c3Ado on proliferation, apoptosis and re-endothelialization in vivo. **A**, Representative cross sections of femoral arteries from mice fed with a control diet only (left), a diet containing 75 μg c3Ado/d (middle) or 150 μg c3Ado/d (right) 4 weeks after dilatation are shown (brown= PCNA, blue= DAPI). The number of proliferating (PCNA positive) cells was determined by dividing the number of PCNA positive cells per section by the total cell number per section (n= 6, *P< 0.05). **B**, Representative cross sections of femoral arteries from mice fed with a control diet only (left), a diet containing 75 μg c3Ado/d (middle) or 150 μg c3Ado/d (right) 4 weeks after dilatation are shown (red= TUNEL, blue= DAPI). The

number of apoptotic cells was determined by dividing the number of TUNEL positive cells per section by the total cell number per section (n= 6, *P< 0.05).

Figure 1

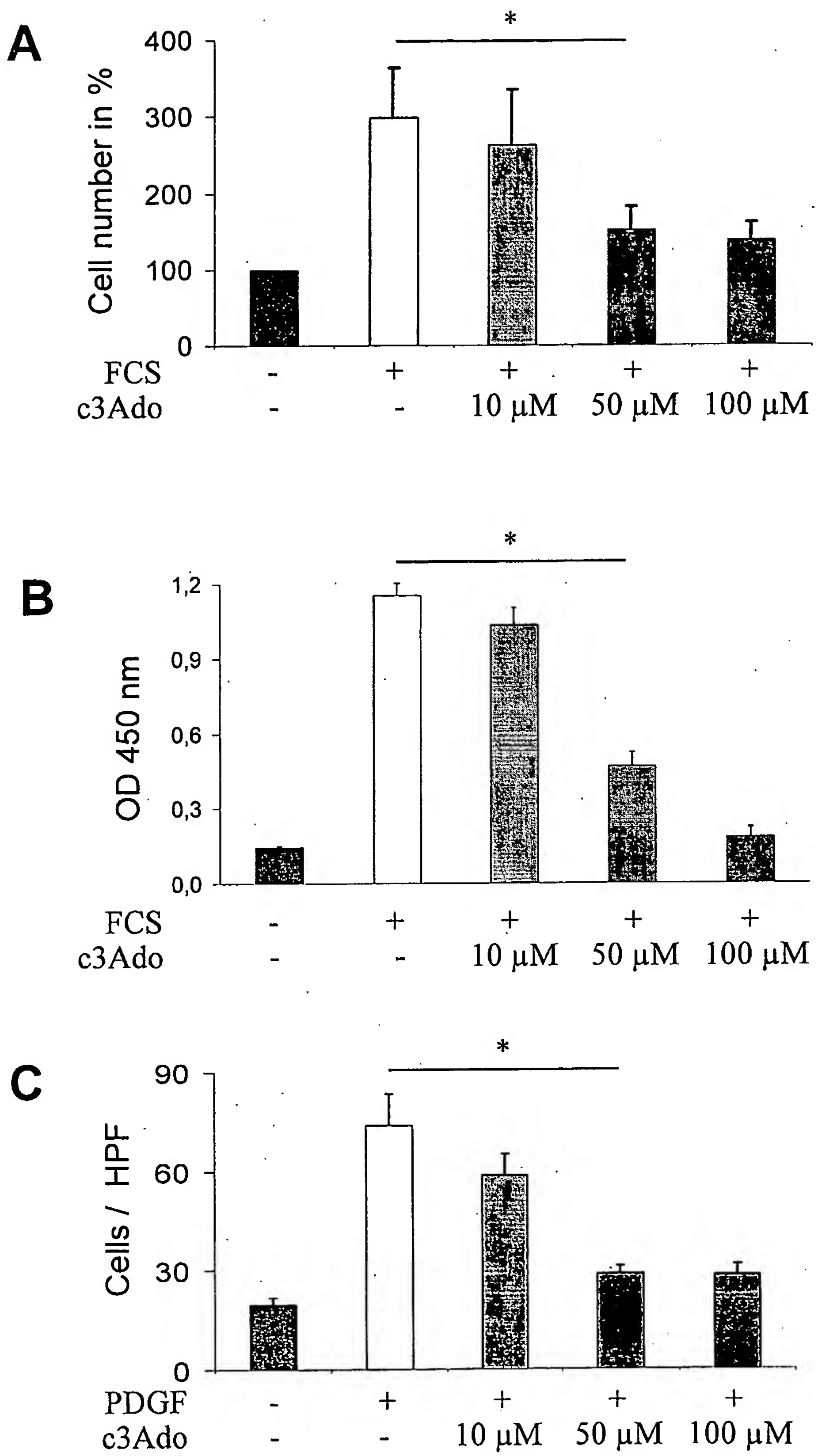
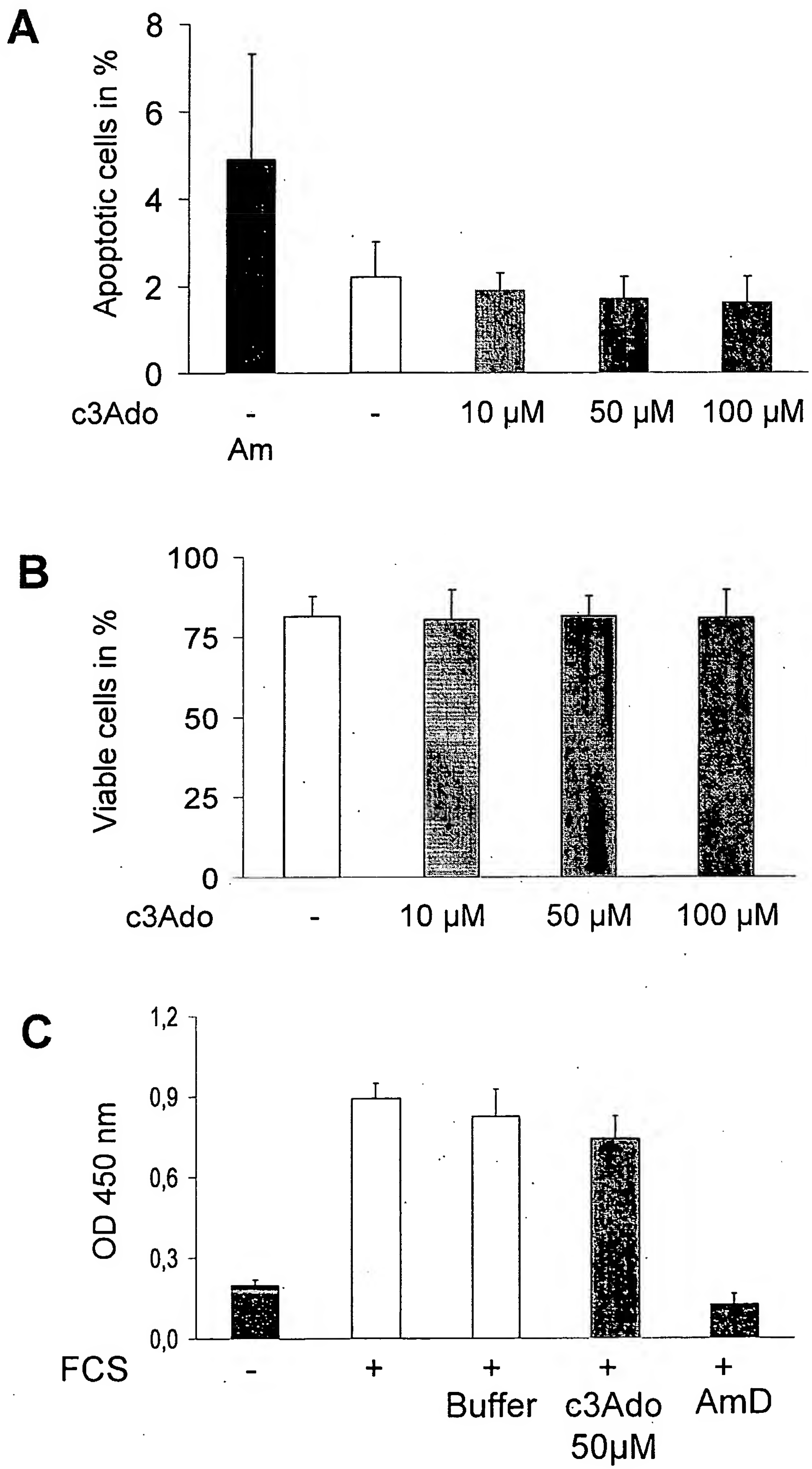

A
*
Cell number in %
400
300
200
100
0
FCS - + + + +
c3Ado - - 10 µM 50 µM 100 µM
B
*
OD 450 nm
1,2
0,9
0,6
0,3
0,0
FCS - + + + +
c3Ado - - 10 µM 50 µM 100 µM
C
*
Cells / HPF
90
60
30
0
PDGF - + + + +
c3Ado - - 10 µM 50 µM 100 µM

Figure 2



A
Apoptotic cells in %
8
6
4
2
0
c3Ado
-
Am
-
10 µM
50 µM
100 µM
B
Viable cells in %
100
75
50
25
0
c3Ado
-
10 µM
50 µM
100 µM
C
OD 450 nm
1,2
0,9
0,6
0,3
0,0
FCS
-
+
+
Buffer
+
c3Ado
50µM
+
AmD

Figure 3

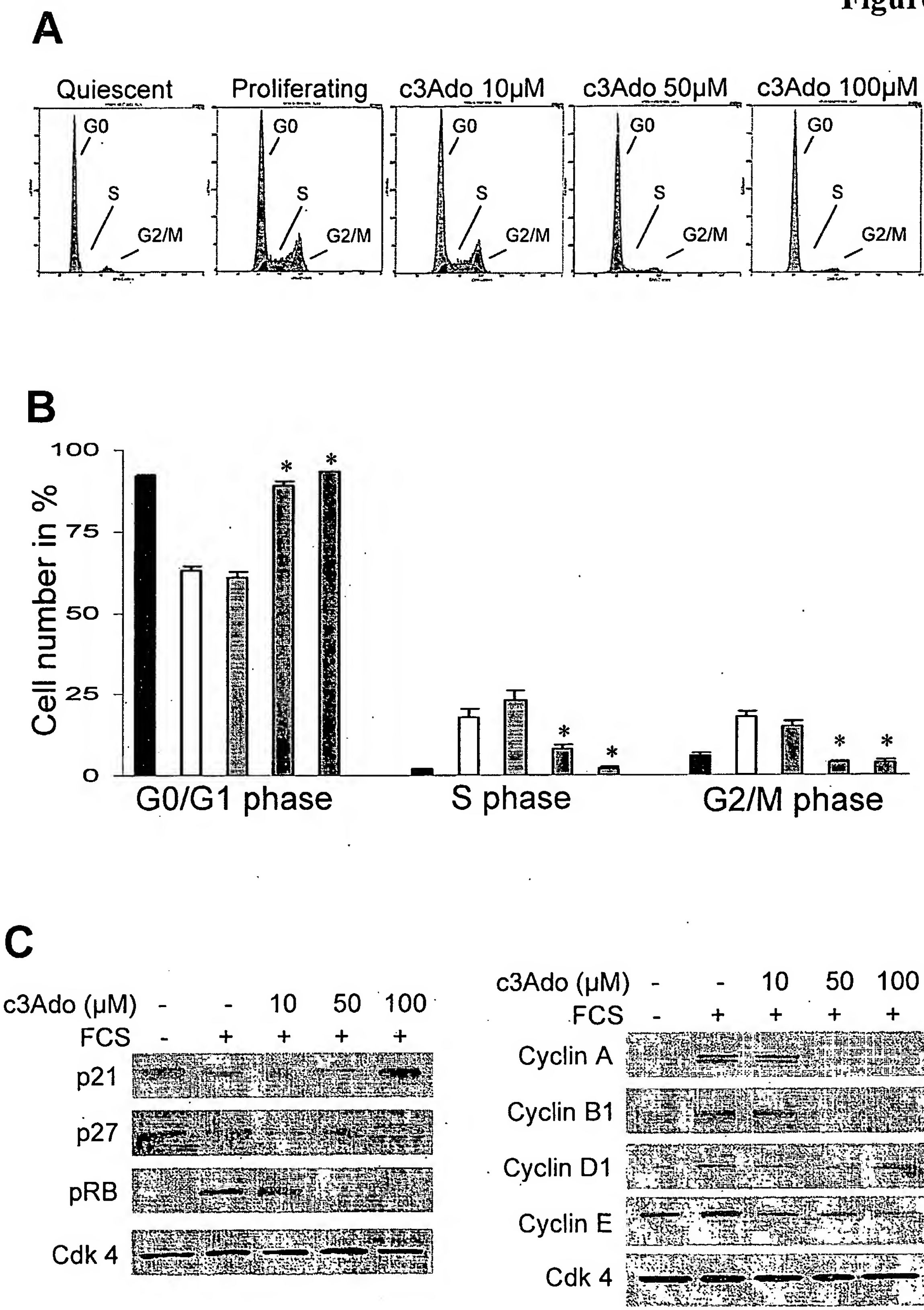

A
Quiescent
Proliferating
c3Ado 10µM
c3Ado 50µM
c3Ado 100µM
G0
S
G2/M
B
100
75
50
25
0
Cell number in %
*
G0/G1 phase
S phase
G2/M phase
C
c3Ado (µM) - - 10 50 100
FCS - + + + +
p21
p27
pRB
Cdk 4
Cyclin A
Cyclin B1
Cyclin D1
Cyclin E
Cdk 4

Figure 4

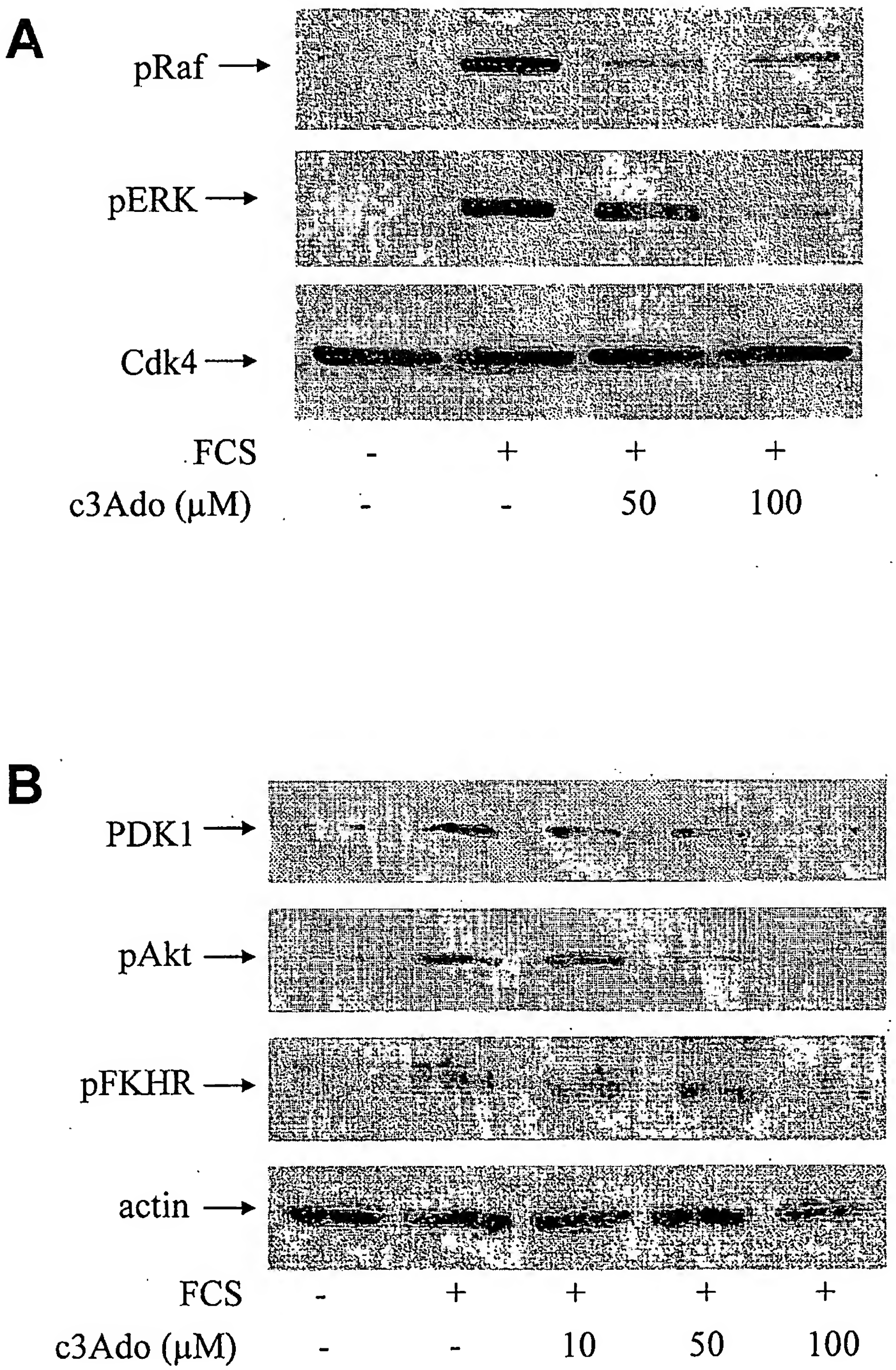

A
pRaf
pERK
Cdk4
FCS - + + +
c3Ado (μM) - - 50 100
B
PDK1
pAkt
pFKHR
actin
FCS - + + + +
c3Ado (μM) - - 10 50 100

Figure 5

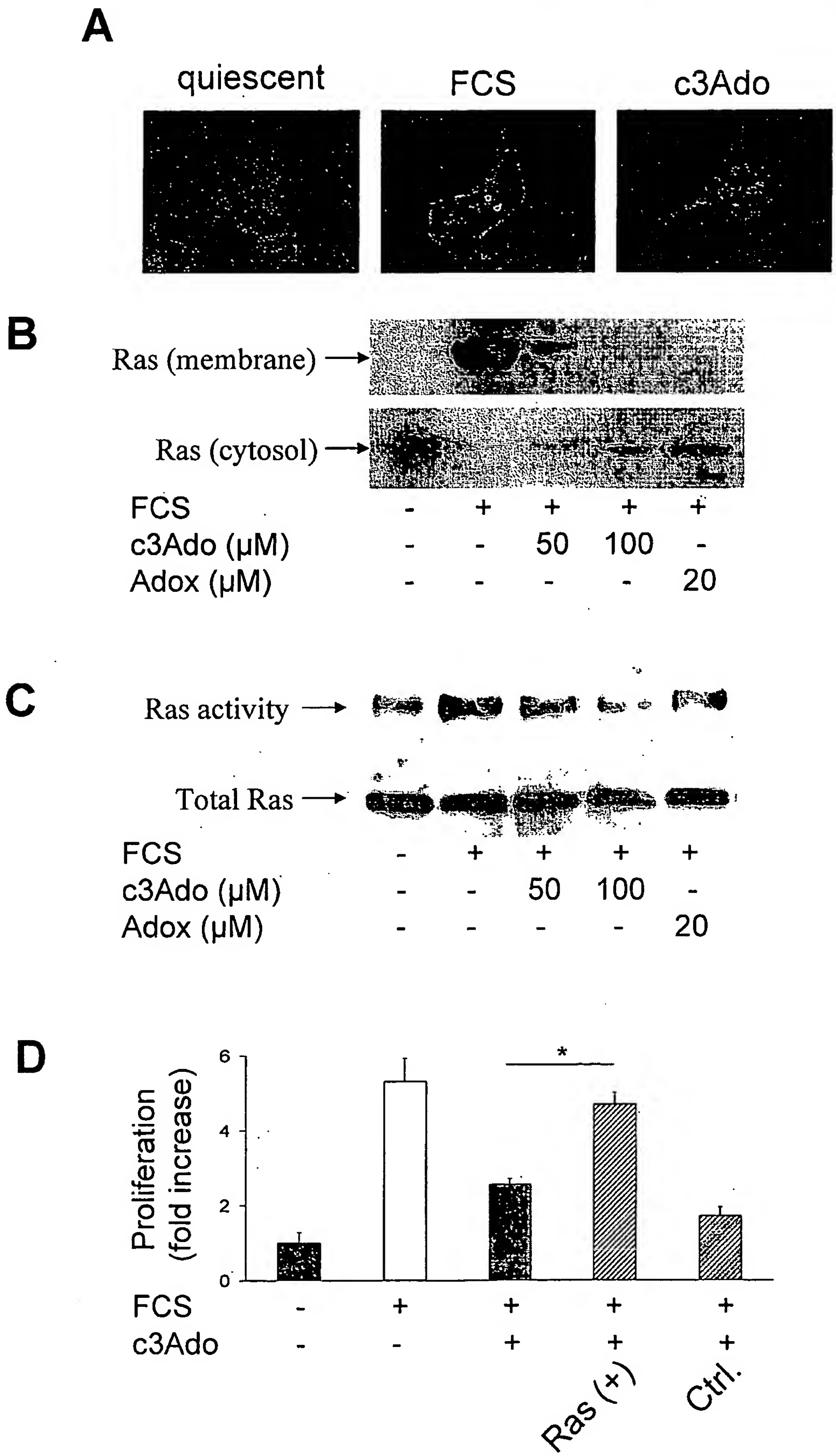

A
quiescent
FCS
c3Ado
B
Ras (membrane)
Ras (cytosol)
FCS - + + + +
c3Ado (µM) - - 50 100 -
Adox (µM) - - - - 20
C
Ras activity
Total Ras
FCS - + + + +
c3Ado (µM) - - 50 100 -
Adox (µM) - - - - 20
D
Proliferation (fold increase)
0
2
4
6
*
FCS - + + + +
c3Ado - - + + +
Ras (+)
Ctrl.

Figure 6

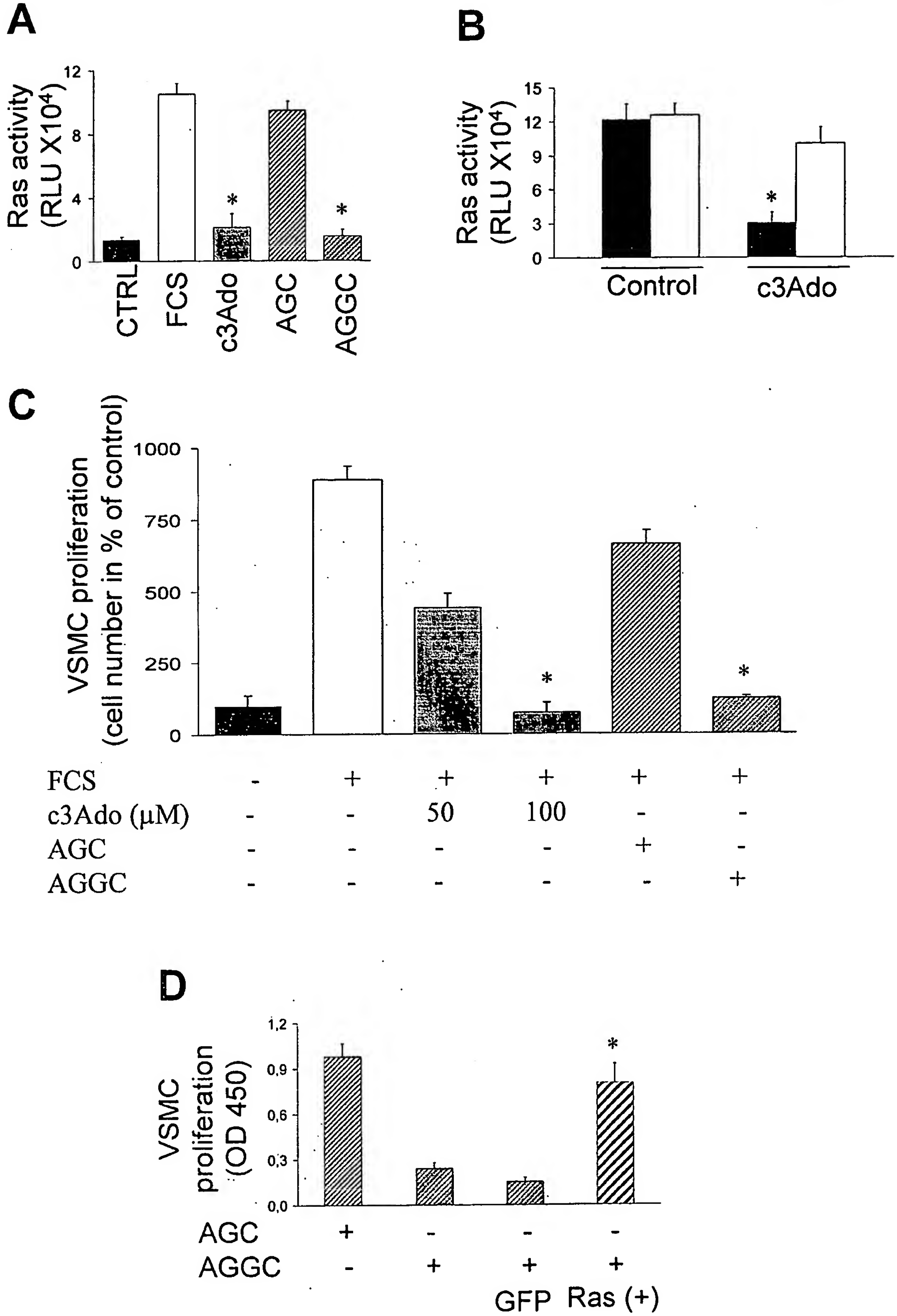

A
Ras activity
(RLU X10^4)
12
8
4
0
*
*
CTRL
FCS
c3Ado
AGC
AGGC
B
Ras activity
(RLU X10^4)
15
12
9
6
3
0
*
Control
c3Ado
C
VSMC proliferation
(cell number in % of control)
1000
750
500
250
0
*
*
FCS - + + + + +
c3Ado (μM) - - 50 100 - -
AGC - - - - + -
AGGC - - - - - +
D
VSMC
proliferation
(OD 450)
1,2
0,9
0,6
0,3
0,0
*
AGC + - - -
AGGC - + + +
GFP
Ras (+)

Figure 7

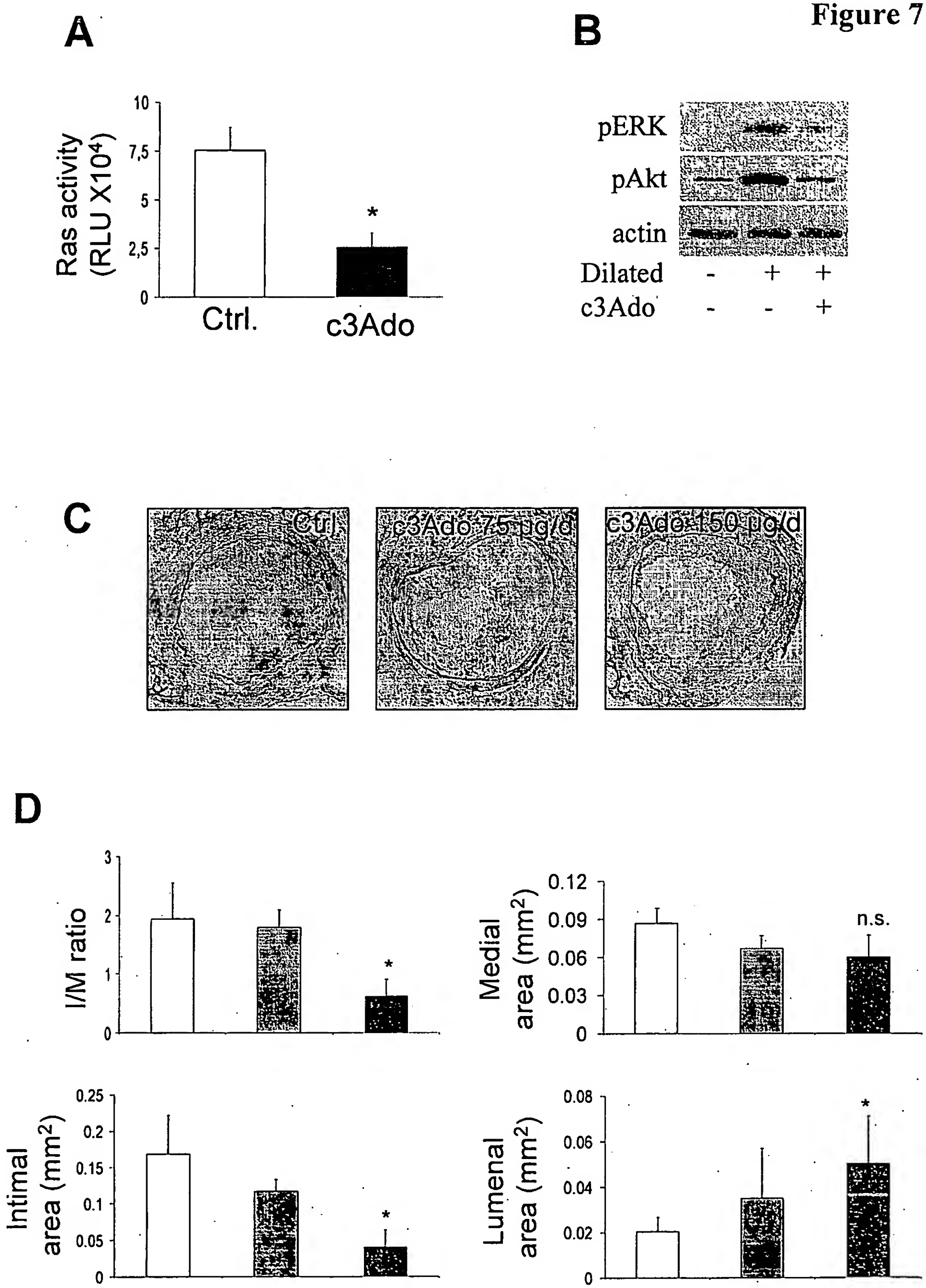

A
Ras activity (RLU X10⁴)
10
7,5
5
2,5
0
*
Ctrl.
c3Ado
B
pERK
pAkt
actin
Dilated - + +
c3Ado - - +
C
Ctrl
c3Ado 75 µg/d
c3Ado 150 µg/d
D
I/M ratio
3
2
1
0
*
Medial area (mm²)
0.12
0.09
0.06
0.03
0
n.s.
Intimal area (mm²)
0.25
0.2
0.15
0.1
0.05
0
*
Lumenal area (mm²)
0.08
0.06
0.04
0.02
0
*

Figure 8